WRS-85D: A Tryptophanyl-tRNA Synthetase Expressed to High Levels in the Developing *Drosophila* Salivary Gland

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In a screen for genes expressed in the *Drosophila* embryonic salivary gland, we identified a tryptophanyl-tRNA synthetase gene that maps to cytological position 85D (*WRS-85D*). *WRS-85D* expression is dependent on the homeotic gene *Sex combs reduced* (*Scr*). In the absence of *Scr* function, *WRS-85D* expression is lost in the salivary gland primordia; conversely, ectopic expression of *Scr* results in expression of *WRS-85D* in new locations. Despite the fact that *WRS-85D* is a housekeeping gene essential for protein synthesis, we detected both *WRS-85D* mRNA and protein at elevated levels in the developing salivary gland. *WRS-85D* is required for embryonic survival; embryos lacking the maternal contribution were unrecoverable, whereas larvae lacking the zygotic component died during the third instar larval stage. We showed that recombinant WRS-85D protein specifically charges tRNA^{Trp}, and *WRS-85D* is likely to be the only tryptophanyl-tRNA synthetase gene in *Drosophila*. We characterized the expression patterns of all 20 aminoacyl-tRNA synthetases and found that of the four aminoacyl-tRNA synthetase genes expressed at elevated levels in the salivary gland primordia, *WRS-85D* is expressed at the highest level throughout embryogenesis. We also discuss the potential noncanonical activities of tryptophanyl-tRNA synthetase in immune response and regulation of cell growth.

INTRODUCTION

How master regulators, such as those encoded by homeotic genes, determine the final structure and physiology of an organism is a long-standing scientific inquiry. We know that homeotic genes are expressed in limited domains along the anterior–posterior body axis where they control which structures develop (Duncan, 1987; Krumlauf *et al.*, 1987; Kaufman *et al.*, 1990; McGinnis and Krumlauf, 1992; Krumlauf, 1994). Mutations in homeotic genes cause structures within a particular segment or segments to be replaced by structures normally found elsewhere. Each homeotic gene encodes one or more related DNA-binding transcription factors (Levine and Hoey, 1988; Scott *et al.*, 1989). Thus homeotic genes control cell fate by regu-

lating transcription of downstream target genes. However, despite a concerted effort to identify these genes in the past several years, only a limited number have been found (Andrew and Scott, 1992; Biggin and McGinnis, 1997; Graba *et al.*, 1997). To identify novel downstream target genes and to learn how homeotic genes control cell fate, we have focused on how *Sex combs reduced* (*Scr*), a *Drosophila* homeotic gene, controls the formation of a relatively simple organ, the larval salivary gland. Our approach has been to identify and characterize candidate target genes based solely on their SCR-dependent expression in the early salivary gland.

Salivary glands provide a simple developmental system to study how early acting regulatory molecules control the assembly of multicellular organs (Campos-Ortega and Hartenstein, 1997). In *Drosophila*, salivary glands start out as two ventrolateral plates of ~100

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cells each in the region of the presumptive posterior head, an area known as parasegment 2 (PS2).[1] No additional cell divisions occur during salivary gland differentiation. Instead, the salivary glands increase in size simply by increasing the volume of individual cells. These cells invaginate and move dorsally and posteriorly, led by cells near the posterior–lateral edge of each plate, leading to the internalization of the salivary glands. By late embryogenesis, salivary gland cells reach the most posterior extent of their migration, reaching to the middle of the third thoracic segment. The salivary gland duct cells are the last to invaginate. These tube-forming cells, which contribute to both the lateral individual ducts and the central common duct, connect the secretory cells of the salivary glands to the larval mouth. The developing salivary gland thus provides a simple system to model cell growth, cell shape changes, cell migration, tube formation, and tissue-specific gene regulation.

Formation of the salivary gland is dependent on the homeotic gene *Scr*. In the absence of *Scr* function, salivary glands do not form, and when *Scr* is expressed everywhere in the embryo, salivary glands form in new locations (Panzer *et al.*, 1992; Andrew *et al.*, 1994; Isaac and Andrew, 1996). However, not every cell that expresses *Scr* becomes salivary gland. Other genes limit the recruitment of *Scr*-expressing cells to a salivary gland fate. The transforming growth factor-*β* signaling cascade limits salivary gland formation to the ventral–lateral regions of PS2 (Panzer *et al.*, 1992; Isaac and Andrew, 1996; Andrew, 1998; Henderson *et al.*, 1998), whereas the localized transcription factors TEASHIRT and ABDOMINAL-B block salivary gland formation in posterior segments when SCR is expressed everywhere (Andrew *et al.*, 1994).

To identify genes expressed in the embryonic salivary gland, we screened several different enhancer trap stock collections (O'Kane and Gehring, 1987; Bellen *et al.*, 1989; Bier *et al.*, 1989; Grossniklaus *et al.*, 1989; Wilson *et al.*, 1989). Enhancer traps are created by the insertion of transposable elements containing the *Escherichia coli lacZ* gene fused to a relatively inactive promoter. Insertion of the transposon within or near enhancers for different genes often results in the expression of *β*-galactosidase (*β*-gal) in patterns that mirror the expression of those genes. This allows us to sample a large portion of the *Drosophila* genome for genes expressed in various tissues by assaying *β*-gal expression in different lines that each harbor a single transposable element. DNA flanking the transposon

insertion site can be recovered using the *E. coli* origin of replication present in the enhancer trap. Mutations in selected genes can be made by mobilizing the transposon and selecting for imprecise excisions. Thus enhancer trap lines provide molecular as well as mutational access to genes selected solely on the basis of their expression within a particular tissue.

We identified 36 different lines in which *β*-gal is expressed in embryonic salivary glands using enhancer trap collections available through three different groups (M. Scott and M. Fuller; A. Spradling; and C. Goodman and G. Rubin). We cloned the gene corresponding to two independent insertions and have shown that it encodes the only *Drosophila* tryptophanyl-tRNA synthetase (*WRS-85D*). Furthermore, we have demonstrated that *WRS-85D* is essential for *Drosophila* development.

MATERIALS AND METHODS

Cloning and Molecular Characterization

DNA flanking the *P-element* inserts in lines *l(3)03559* and *l(3)04410* was obtained by plasmid rescue (Hamilton and Zinn, 1994) and used to isolate both genomic and cDNA *WRS-85D* clones (Zinn *et al.*, 1988; Tamkun *et al.*, 1991). Library screening, plasmid, phage, and genomic DNA preparations, subcloning, and labeling of radioactive probes were performed as described (Maniatis *et al.*, 1989). The 2.6- and 1.6-kb cDNAs were subcloned into pGEM7Zf to yield plasmids pPS10.1 and 11.1, respectively. The developmental Northern blot was prepared as described (Henderson and Andrew, 1998). DNA sequencing was performed at the Johns Hopkins University Core DNA Analysis Facility and also as described (Isaac and Andrew, 1996). Sequence alignments were done using the CLUSTAL X (Higgins, 1993) and MacBoxshade programs (Baron, 1997, MacBoxshade; http://www.netaxs.com/~jayfar/mops.html.).

Reduced Stringency Genomic Southern Blot

Plasmid pPS10.1 was digested with *Eco*RI, and the 660-bp fragment encoding the N-terminal 200 residues of *WRS-85D* was isolated and used to probe a blot of Oregon R (wild-type) *Drosophila melanogaster* genomic DNA under reduced stringency hybridization conditions (42°C, 5× SSC, 30% formamide) (Laird *et al.*, 1969).

Polytene Chromosome In Situ Hybridizations

Polytene chromosome in situ hybridizations were done by the procedure of Pardue (1994) omitting the RNase treatment and acetylation steps and using the Vectastain kit (Vector Laboratories, Burlingame, CA) for HRP signal detection.

Antibodies, Embryo Staining, and Whole-Mount In Situ Hybridization

The *β*-gal mouse mAb was obtained from Promega (Madison, WI). The rat polyclonal antisera to WRS-85D was raised against an N-terminal 200-residue peptide. The PCR (Saiki *et al.*, 1985) was used to amplify the most 5′ 600 bp of the ORF of WRS-85D using as template clone pPS10.1, which contains the 2.6-kb *WRS-85D* cDNA clone, with forward primer (5′-GGGGCTCGAGAATGGCGGA-CACCAAGGAG) and reverse primer (5′-TGCCCTTGACCTGAT-TGA). The resulting product was digested with *Xho*I and *Eco*RI and subcloned into the *Xho*I–*Eco*RI sites of pTrcHisB (Invitrogen, Carls-
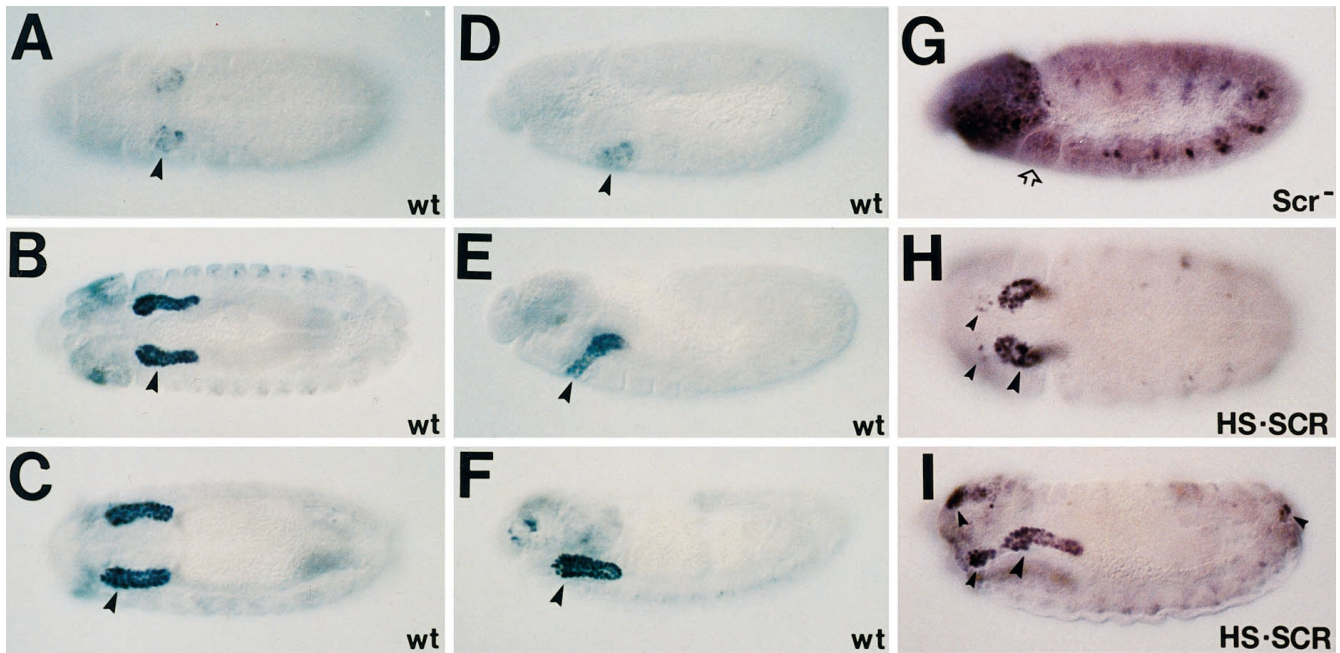
Figure 1. Expression of enhancer traps in WRS-85D is observed at high levels in the salivary gland and is under the control of the homeotic gene Scr. Embryos have been immunostained with an mAb to β-gal. Embryos in A–C, and H are ventral views; embryos in D–G and I are lateral views. Embryos in A–F show staining dependent on the *P-element* insertion in the stock l(3)03559 in a wild-type genetic background. In addition to the high-level expression of β-gal in the salivary gland, we also detected expression in the head sensory sensilla that will form the dorsal and ventral organs (F) (Campos-Ortega and Hartenstein, 1997). (G) Immunostaining from the *P-element* stock in an embryo missing Scr function (an Scr⁴ homozygote). Note the loss of expression in the salivary gland primordia even when the embryo is significantly overstained to demonstrate both head and PNS staining. (H and I) β-Gal staining in an l(3)03559 embryo where SCR is expressed everywhere under the control of an induced heat-shock promoter (HS·SCR). Note the ectopic β-gal expression in ventrolateral cells of PS1 in H and in PS0, PS1, and PS14 in I. An identical profile of β-gal expression is observed in embryos carrying the l(3)04410 insertion (our unpublished results).

bad, CA) downstream of and in frame with the His$_6$ tag. This construct, pPS12.2, was transformed into BL21(DE3) cells (Studier *et al.*, 1990). A 500-ml culture of the pPS12.2-transformed cells was grown to an OD$_{600}$ of 0.6, isopropyl-1-thio-β-ᴅ-galactopyranoside was added to a final concentration of 0.1 mM, and the cells were grown for an additional 5.5 h to induce expression of the fusion protein. WRS-85D protein was purified from the induced cells by isolation of inclusion bodies (Rio *et al.*, 1986) followed by Ni-NTA (Qiagen, Santa Clarita, CA) affinity chromatography (Invitrogen). Rat polyclonal antisera were raised against 1.3 mg of the Ni-NTA–purified protein (Covance Research Products, Denver, PA).

Embryo fixation and staining were performed as described, except Bouin's solution (Sigma, St. Louis, MO) was used as fixative for WRS-85D immunostaining. Homozygous mutant embryos were identified by the absence of staining with α-β-gal, which stains the nonmutant embryos that carry a balancer chromosome marked with an *ftz-lacZ* insert on TM3. Immunostained embryos were mounted in methyl salicylate (Sigma).

Whole-mount in situ hybridization to detect embryonic mRNA accumulation was performed as described by Lehmann and Tautz (1994). Embryos were mounted in 70% glycerol to limit diffusion of the alkaline phosphatase reaction products.

Both immunostained embryos and embryos used for whole-mount in situ hybridization were visualized and photographed on a Zeiss (Thornwood, NY) Axiophot microscope using Normarski optics. Ektar print film (Eastman Kodak, Rochester, NY) was used for photography.

Cuticle Preparations

Cuticle preparations were as described in Andrew *et al.* (1994). Preparations were examined using both phase and dark-field optics and photographed with TMAX 100 print film (Kodak).

Fly Stocks, Excisional Mutagenesis, Lethal Phase Determination, and Generation of Germ Line Clones

The wild-type flies used in all experiments were Canton S or Oregon R. The *P-element* insertion alleles, l(3)03559 and l(3)04410, are described in FlyBase (http://www.flybase.org). Excisional mutagenesis to revert the lethality and to create additional alleles of *WRS-85D* was performed as described (Hamilton and Zinn, 1994). The lethal phase for *WRS-85D* zygotic loss of function was determined by collecting embryos and counting the number of balancer (*Tubby*) and nonbalancer (non-*Tubby*) animals at each developmental stage.

To obtain embryos missing both maternal and zygotic function of *WRS-85D*, we generated homozygous mutant germ line clones using the dominant female sterile P[ovo^D1] flippase (FLP)–FRT recombination technique (Chou and Perrimon, 1996). Females with FRT^82B and a *WRS-85D* loss-of-function allele on the third chromosome were crossed with males with FRT^82B and P[ovo^D1] on the third chromosome and one copy of hs-flippase (hsFLP) on the X chromosome. hsFLP-induced germ line clone-bearing females were crossed to males heterozygous for a loss-of-function mutation in *WRS-85D*, and embryos were collected.
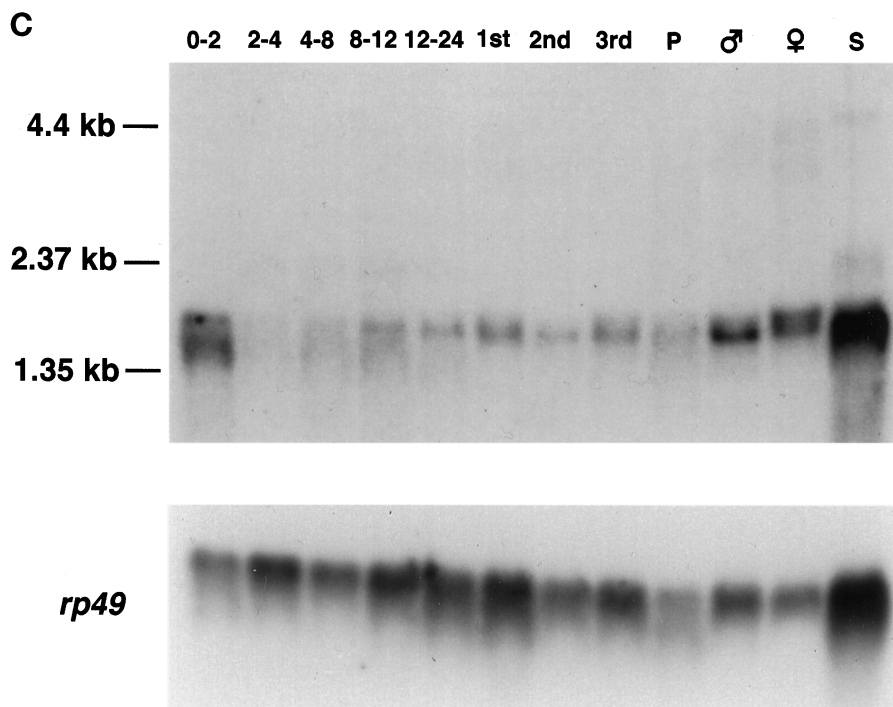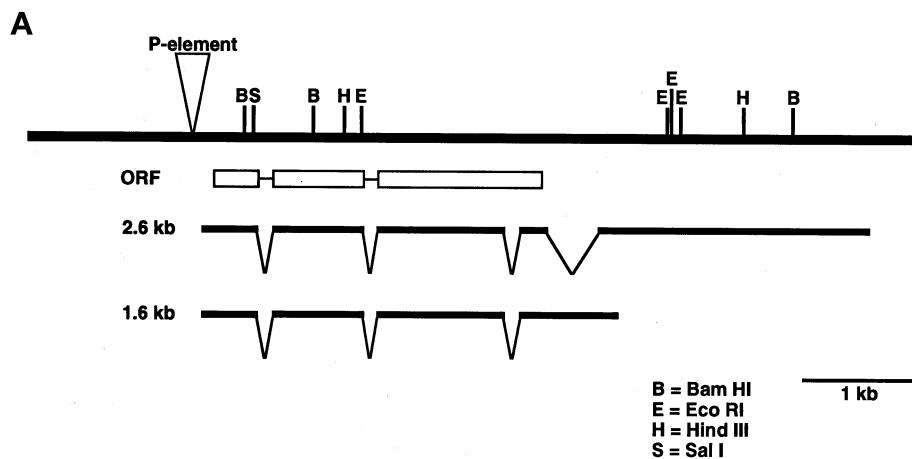
A

C

Figure 2. WRS-85D encodes a protein homologous to mammalian tryptophanyl-tRNA synthetases. (A) Genomic map of *WRS-85D* showing insertion site of *I(3)03559/l(3)04410*, cDNA mapping, and intron–exon boundaries. (B) Alignment between *WRS-85D* and mammalian TrpRS. Residues that are identical are black; residues that are similar are gray. The HVGH and KMSAS signature motifs are indicated with black lines above the sequence. Sequences for two WRS-85D splice forms are available at GenBank (accession numbers BankIt250528 AF125156 and BankIt250542 AF125157). (C) Developmental Northern blot showing expression profile throughout development. 0–2, 2–4, 4–8, 8–12, and 12–24, hours of embryogenesis; 1st, 2nd, and 3rd, three larval stages; P, pupae; S, *Drosophila* Schneider cells. The same Northern blot is shown hybridized to *rp49* probe as a control for loading.

Purification of WRS-85D for Aminoacylation Activity Assay

Clone pPS10.1 was digested with *Eco*RI, and the fragment from position 601-1527 of the cDNA, which contains the remainder of the WRS-85D ORF, was subcloned downstream of and in frame with the 5′ region of *WRS-85D* in pPS12.2. This construct, pPS17.6, was transformed into BL21(DE3). Expression of the fusion protein was induced as described above. The cells were resuspended in NBB (Invitrogen), sonicated, and centrifuged at 26,000 × *g*. The supernatant was then purified by Ni-NTA affinity chromatography (Invitrogen). The pooled fractions were dialyzed against 100 mM Tris, pH 8.0, 1 mM EDTA, 10% glycerol. Protein concentration was determined using the BCA assay (Pierce, Rockford, IL). An identical protocol was applied to BL21(DE3) cells transformed with the vector pTrcHisB to obtain mock-purified protein as a negative control.

Determination of Aminoacylation Activity

Twenty-three micrograms of purified WRS-85D protein were combined with 142 mM Tris, pH 8.0, 1.42 mM EDTA, 15 mM MgOAc, 0.05 mg/ml BSA, 0.1 mM [^{14}C]L-tryptophan (54 mCi/mmol; DuPont NEN, Wilmington, DE), 7 mg/ml total tRNA from Brewer's yeast (Boehringer Mannheim, Indianapolis, IN) and 4.2% glycerol in a 60-μl reaction volume (Bange *et al.*, 1992). ATP was added to 8 mM to initiate the reaction, and the mixture was incubated at 30°C. Aliquots of 9 μl were removed at various time points, precipitated with 5% trichloroacetic acid (TCA)/0.5% Trp, spotted on GF/C filters (Whatman, Clifton, NJ), and washed with 5% TCA. Radioactivity retained on the filters was quantitated with a scintillation counter. Twenty micrograms of mock-purified protein from pTrcHisB-transformed cells was used in parallel experiments as a negative control, and 1.1 μl of reticulocyte lysate (Promega) were used in parallel experiments as a positive control. Each experiment

B

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WRS-85D  ........................................MADTKETVVEGVEA
Bovine   MADMSNGEQGCGSPLELFHSIAAQGELVRDLKARNAAKDEIDSAVKMLLS
Human    ...MPNSEP..ASLLELFNSIATQGELVRSLKAGNASKDEIDSAVKMLVS
Mouse    MADMPSGES.CTSPLELFNSIATQGELVRSLKAGNAPKDEIDSAVKMLLS
Rabbit   MADVTNGER.CASPQELFSSIAAQGELVKSLKARKAPKEEIDSAVKMLLS


WRS-85D  LTLN....G....KPDAEPVETGTDAQAQEGATAPTEDVVDPWNVASSND
Bovine   LKTSYKAATGEDYKVDCPPGDPAPESGEGLDATEADEDFVDPWTVQTSSA
Human    LKMSYKAAAGEDYKADCPPGNPAPTSNHGPDATEAEEDFVDPWTVQTSSA
Mouse    LKMSYKAAMGEEYKAGCPPGNPTAGRNCDSDATKASEDFVDPWTVRTSSA
Rabbit   LKTSYKEAMGEDYKADCPPGNSTPDSHGDPFAVDDKEDFVDPWTVRTSSA


WRS-85D  AGVDYDKLIKRFGSSKIDEELIARFEKITGKPAHHFLRRGMFFSHRDLHT
Bovine   KGIDYDKLIVRFGSSKIDKELVNRIERATGQRPHRFLRRGIFFSHRDMHQ
Human    KGIDYDKLIVRFGSSKIDKELINRIERATGQRPHHFLRRGIFFSHRDMNQ
Mouse    KGIDYDKLIVQPGSSKIDKELINRIERATGQRPHRFLRRGIFFSHRDMNQ
Rabbit   KGIDYDKLIVQFGSSKIDKELVNRIERATGQRPHRFLRRGIFFSHRDMNQ


WRS-85D  ILTLREQGKPFYLYTGRGPSSGSLHVGHLVPFIMTKWLQETFDVPLVIQL
Bovine   ILDAYENKKPFYLYTGRGPSSEAMHVGHLIPFIFTKWLQDVFNVPLVIQM
Human    VLDAYENKKPFYLYTGRGPSSEAMHVGHLIPFIFTKWLQDVFNVPLVIQM
Mouse    ILDAYENKKPFYLYTGRGPSSEAMHLGHLVPFIFTKWLEDVFNVPLVIQM
Rabbit   VLDAYENKKPFYLYTGRGPSSEAMHVGHLIPFIFTKWLQDVFDVPLVVQM


WRS-85D  TDDEKTLWKDLKVEDAIKLGRENAKDIVAIGFDVNKTFIFNNLEFVGKCP
Bovine   TDDEKYLWKDLTLDQAYGYAVENAKDIT.CGFDINKTFIFSDLDYMGMSP
Human    TDDEKYLWKDLTLDQAYSYAVENAKDIIACGFDINKTFIFSDLDYMGMSS
Mouse    SDDEKYLWKDLTLEQAYSYTVENAKDIIACGFDINKTFIFSDLEYMGQSP
Rabbit   SDDEKYLWKDLTLEQVYGYTLENAKDIIACGFDVNKTFIFSDLDYMGMSP


WRS-85D  AMYQNIIRIQKCVTFNQVKGIFGFGDSDIIGKIGFPAAQAAPAISSTFPF
Bovine   GFYKNVVKIQKHVTFNQVKGIFGFTDSDCIGKISFPAIQAAPSFSNSFPQ
Human    GFYKNVVKIQKHVTFNQVKGIFGFTDSDCIGKISFPAIQAAPSFSNSFPQ
Mouse    GFYRNVVKIQKHVTFNQVKGIFGFTDSDCIGKSSFPAVQAAPSFSNSFPK
Rabbit   GFYKNVVKIQKHVTFNQVKGIFGFTDSDCIGKISFPAIQAAPSFSNSFPQ


WRS-85D  IFGNR.KVHCLIPCAIDQDPYFRMTRDVAPRLGFPKCALIHSTFFPALQG
Bovine   IFRDRTDVQCLIPCAIDQDPYFRMTRDVAPRIGYPKPALLHSTFFPALQG
Human    IFRDRTDIQCLIPCAIDQDPYFRMTRDVAPRIGYPKPALLHSTFFPALQG
Mouse    IFRDRTDIQCLIPCAIDQDPYFRMTRDVAPRIGHPKPALLHSTFFPALQG
Rabbit   IFHGQADIQCLIPCAIDQDPYFRMTRDVAPRIGYPKPALLHSTFFPALQG


WRS-85D  AKTKMSASDQNSAVYLTDTPKQIKNKINKYAFSGGRVTVEEHRKLGGVPE
Bovine   AQTKMSASDPNSSIFLTDTAKQIKTKVNKHAFSGGRDTVEEHRQFGGNCD
Human    AQTKMSASDPNSSIFLTDTAKQIKTKVNKHAFSGGRDTIEEHRQFGGNCD
Mouse    AQTKMSASDPNSSIFLTDTAKQIKSKVNKHAFSGGRDTVEEHRQFGGNCE
Rabbit   AQTKMSASDPNSSIFLTDTAKQIKTKVNKHAFSGGRDTIEEHRQFGGNCD


WRS-85D  VDVSYQLLKFFLEDDAKLEEVRVAYSKGEMLTGEIKKLAVETLTPIVEQH
Bovine   VDVSFMYLTFFLEDDDKLEQIRRDYTSGAMLTGELKKELIEVLQPLIAEH
Human    VDVSFMYLTFFLEDDDKLEQIRKDYTSGAMLTGELKKALIEVLQPLIAEH
Mouse    VDVSFMYLTFFLEDDDRLEQIRKDYTSGAMLTGELKKTLIDVLQPLIAEH
Rabbit   VDVSFMYLTFFLEDDDKLEQIRKDYSSGAMLTGELKKELIDVLQPLVAEH


WRS-85D  QAARKLITDEVLDKYFELRPLKFGS.......
Bovine   QARRKEVTDEIVKEFMTPRKLSYDFQ......
Human    QARRKEVTDEIVKEFMTPRKLSFDFQ......
Mouse    QARRKAVTEETVKEFMTPRQLSFHFQCFCFDT
Rabbit   QARRKEVTDEMVKEFMTPRQLCFHVQ......
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Figure 2 (cont).

was independently repeated three times. Experiments were also carried out in triplicate using 0.1 mM [^{14}C][scsp]l-leucine to control for enzyme specificity.

RESULTS

Tryptophanyl-tRNA Synthetase (WRS) Is Expressed to High Levels in the Drosophila Salivary Gland

In an enhancer trap screen for genes expressed in the early *Drosophila* salivary gland, two independent insertions at cytological position 85D7,8 were identified, *l(3)03559* and *l(3)04410*. Both lines presented very high levels of β-gal expression in the secretory portion of the salivary gland with additional low-level staining in a subset of cells in the peripheral nervous system (PNS) (Figure 1, A–F). The salivary gland expression of β-gal in these lines was SCR dependent. In *Scr* null embryos, we did not detect β-gal expression in the salivary gland primordia even when embryos were overdeveloped to show strong staining in the PNS (Figure 1G). In embryos that carried an HS-SCR transgene, a construct containing the *Hsp70* enhancer fused to an *Scr* cDNA (Zeng *et al.*, 1993), and were heat shocked, we observed ectopic β-gal expression in cells derived from PS0, PS1, and PS14 (Figure 1, H and I). Cells expressing ectopic β-gal are found at approximately the same dorsal–ventral position as salivary gland primordial cells in PS2 (Figure 1H). Furthermore, β-gal–expressing cells derived from PS1 often invaginate and remain attached to the salivary gland cells of PS2 (Figure 1I), suggesting that these cells have adopted a salivary gland fate.

To identify the gene corresponding to the enhancer trap lines, we used plasmid rescue to isolate genomic DNA flanking one side of the P-element insertion site. In situ hybridization to *Drosophila* polytene chromosomes localized the genomic DNA to the 85D7,8 region of chromosome 3, consistent with the position of the P-element insertions (our unpublished results). We sequenced the junction between the genomic DNA and the P-element and found that in both *l(3)03559* and *l(3)04410*, the P-element had inserted into the identical position in the genome, 103 bp upstream of an ORF. Therefore, we consider *l(3)03559* and *l(3)04410* to be equivalent.

The plasmid rescue DNA was used to isolate several cDNAs ranging in size from 1.2 to 2.6 kb. The 2.6-kb cDNA clone was sequenced in its entirety. Conceptual translation of this cDNA revealed a 430-residue ORF with strong homology to mammalian tryptophanyl-tRNA synthetases (TrpRS/WRS) (BLAST at National Center for Biotechnology Information, Bethesda, MD; Altschul *et al.*, 1997). *WRS-85D* and mammalian TrpRS are 53% identical and 63% similar (Figure 2B). Tryptophanyl-tRNA synthetases covalently link tryptophan to its cognate tRNA before protein translation. TrpRS is a class I aminoacyl-tRNA synthetase, whose members contain the "HVGH" and "KMSAS" signature sequences (Meinnel *et al.*, 1995; Arnez and Moras, 1997). The HVGH and KMSAS motifs are conserved in WRS-85D, supporting its identification as a tryptophanyl-tRNA synthetase.

To characterize the expression pattern of *WRS-85D*, we carried out both developmental Northern analysis and in situ hybridizations to whole-mount embryos. We detected several *WRS-85D* transcripts throughout development by Northern blot analysis (Figure 2C). The most abundant transcripts are estimated to be 1.65 and 1.8 kb, based on the migration of RNA standards. Because *WRS-85D* transcripts were detected in 0- to 2-h embryos, the mRNA is most likely to be provided maternally, because zygotic transcription does not begin until ~2.5 h after egg laying. We also detected *WRS-85D* transcripts in *Drosophila* Schneider tissue culture cells.

To examine the spatial expression pattern of *WRS-85D*, we performed in situ hybridizations on whole-mount embryos. As shown in Figure 3, A–D, we detected abundant transcript levels in the secretory portion of the salivary gland primordia. This high level of expression in salivary gland secretory cells was visible throughout embryogenesis and was consistent with the expression of β-gal from the two *P-element* insertions in the *WRS-85D* gene. We were unable to detect *WRS-85D* transcripts in the PNS probably because of differences in the sensitivity of β-gal versus RNA detection or because of the response of the enhancer trap line to enhancers from other nearby gene(s). At present, we cannot distinguish between these two possibilities. However, consistent with the β-gal expression from the two enhancer trap lines, salivary gland expression of *WRS-85D* is SCR dependent (our unpublished results).

We generated antisera to the WRS-85D protein and used it to immunostain whole-mount embryos (Figure 3, E–H). We detected elevated expression of the WRS-85D protein in the salivary gland and its primordia. However, we also detected a global expression pattern of relatively high levels of the protein. We propose that the protein detected in all cells may be partially due to translation of maternally provided *WRS-85D* transcripts that would be distributed at equivalent levels to all cells of the embryo. Low levels of zygotically transcribed *WRS-85D*, visible when the detection step of our in situ hybridization reactions was allowed to continue for several hours, might also contribute to overall protein levels. So, although *WRS-85D* expression is not limited to the salivary gland secretory cells, it is much higher in these cells than in other embryonic tissues.

To determine whether *WRS-85D* is the only TrpRS gene in *Drosophila*, we first performed a genomic Southern analysis using four different restriction enzymes and a probe encoding the N-terminal 200
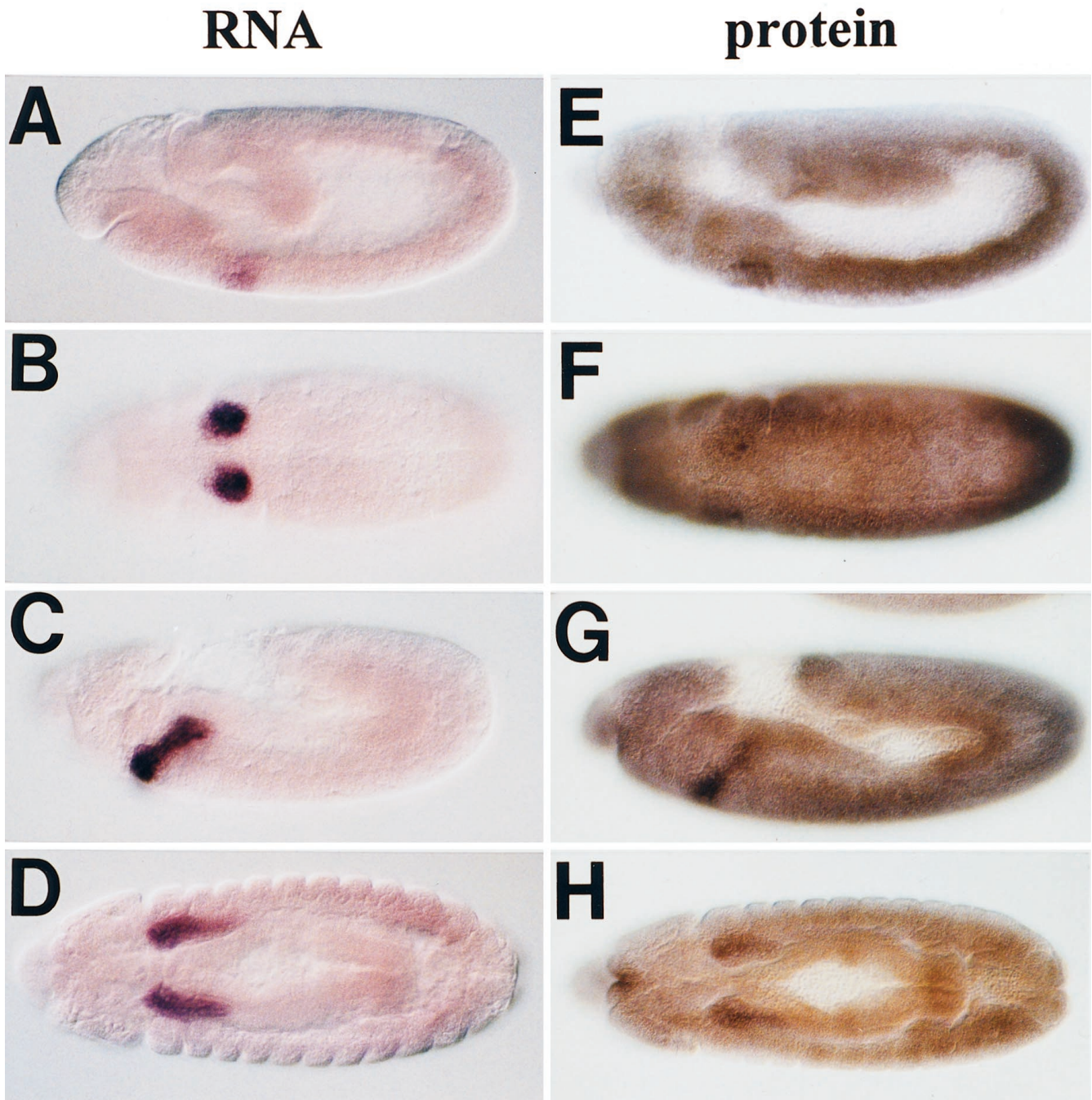
RNA

protein

Figure 3. WRS-85D RNA and protein show similar patterns of accumulation. (A–D) Embryos that have been hybridized with a *WRS-85D* antisense digoxygenin-labeled RNA probe. (E–H) show embryos that have been immunostained with polyclonal antisera raised against the WRS-85D protein. (A, C, E, and G) Lateral views. (B, D, F, and H) Ventral views. Note the similarity in the patterns observed with the *WRS-85D* transcript and protein.

residues, which include the conserved HVGH ATP-binding motif. Even under reduced stringency hybridization conditions, the probe hybridized only to DNA bands that corresponded in size to the *WRS-* *85D* locus (our unpublished results). Additionally, our search of the Berkeley *Drosophila* Genome Project Expressed Sequence Tags database (http://www.fruitfly.org/EST) identified two new WRS

cDNAs (LD24552 and GH06221). The ORF sequences available from these two clones were identical matches to the *WRS-85D* sequence (our unpublished results). Finally, all the cDNAs isolated in our screen hybridized to a single, resolvable locus, *WRS-85D* at 85D7,8, on polytene chromosomes (our unpublished results). Based on these data, we conclude that *WRS-85D* is the only tryptophanyl-tRNA synthetase gene in *D. melanogaster*.

WRS-85D Has tRNA^Trp Charging (Aminoacylation) Activity

To demonstrate that *WRS-85D* encodes a functional tryptophanyl-tRNA synthetase, we expressed and purified WRS-85D from bacteria as an N-terminal His_6-tagged fusion protein. The fusion protein, purified by Ni-NTA affinity chromatography, migrated as a polypeptide with an apparent molecular mass of 55 kDa on SDS-PAGE gels, close to its predicted mass of 51 kDa (our unpublished results). If WRS-85D possesses tRNA^Trp charging activity, then we would expect [^{14}C]L-tryptophan to be covalently linked to tRNA^Trp in an enzymatically dependent manner. When we incubate [^{14}C]L-tryptophan with recombinant WRS-85D, the covalent linkage of [^{14}C]L-tryptophan to tRNA^Trp can be measured as acid-precipitatable counts on GF/C filters.

We observed increasing amounts of TCA-precipitatable counts over the time course of the assay, indicating that yeast tRNA was tryptophanylated by WRS-85D (Figure 4A). This activity was substrate dependent, because only a background level of counts was obtained when using [^{14}C]L-leucine instead of [^{14}C]L-tryptophan (Figure 4B). As expected, the reaction was ATP dependent; omission of ATP resulted in a complete loss of activity (our unpublished results). The tRNA^Trp charging activity could be assigned specifically to WRS-85D, because mock-purified protein from bacteria containing only the expression vector pTrcHisB gave a low level of counts that did not increase over time. Therefore, beyond the significant sequence homology to other TrpRS, WRS-85D tryptophanylates tRNA and is a tryptophanyl-tRNA synthetase.

WRS-85D Is an Essential Gene

Sequencing the plasmid rescue DNA from *l(3)03559* and *l(3)04410* revealed that the P-element had inserted, in both cases, 103 bp upstream of the *WRS-85D* ORF. Both lines are homozygous lethal. To test whether the lethality is due to the insertions and to generate additional alleles of the gene, we initiated an excisional mutagenesis screen selecting for the loss of the *rosy*$^+$ (*ry*$^+$) eye color marker contained within the P-element (Hamilton and Zinn, 1994). From 115 independent lines, we obtained 43 lethal lines, 22 semile-

thal lines, and 29 homozygous viable lines. The recovery of 29 viable lines associated with loss of the P-element suggests that the lethality in both *l(3)03559* and *l(3)04410* is due to the insertion of the P-element disrupting *WRS-85D* function and not due to a mutation at another site.

We used complementation tests among the lethal excision lines derived from *l(3)03559* and *l(3)04410* to identify a set of potential *WRS-85D* alleles. Based on immunostaining of homozygous *WRS-85D* mutant embryos with anti-WRS-85D antisera, neither the original insertion alleles *l(3)03559* and *l(3)04410* nor the lethal alleles that failed to complement the original insertions make detectable levels of WRS-85D in the salivary glands (Table 1). However, WRS-85D levels in the salivary glands in embryos from the viable excisant lines were equivalent to the levels observed in wild-type embryos. These results establish that loss-of-function mutations in *WRS-85D* are lethal.

Immunostaining of embryos homozygous mutant for the lethal *WRS-85D* alleles with antibodies to nuclear (dCREB-A) or lumenal (CRUMBS) salivary gland markers revealed no overt defects in the salivary gland (our unpublished results). Because these homozygous mutants do not survive to adulthood, we determined the lethal phase by using the *Tubby*-containing balancer chromosome TM6B to distinguish the homozygous *WRS-85D* mutant larvae (non-*Tb*) from their heterozygous (*Tb/+*) or homozygous (*Tb/Tb*) balancer siblings. *Tb* heterozygotes and homozygotes are short and squat relative to non-*Tb* larvae and pupae. Animals homozygous for two of the four tested *WRS-85D* alleles died during the larval stages, whereas animals homozygous for the other two alleles died during the larval–pupal transition (our unpublished results). We also examined the cuticles of homozygous mutant larvae and found no overt defects (our unpublished results).

Because *WRS-85D* encodes a housekeeping gene, we were surprised that the homozygous mutant animals survived embryogenesis and the early larval stages. However, results from the Northern blot analysis and WRS-85D immunostaining indicate that *WRS-85D* is maternally contributed. To determine whether this maternal contribution is allowing the *WRS-85D* mutant animals to survive beyond embryogenesis, we used the FLP-FRT system to generate germ line clones (Chou and Perrimon, 1996) that removed the maternal contribution of *WRS-85D* transcripts. Although we collected ~3200 females from larvae heterozygous for two protein-null alleles that had been subjected to germ line clone induction, we failed to obtain any eggs from these animals. Thus, *WRS-85D* must be essential in oogenesis and is likely to be essential in all cells. This result suggests that the maternal contribution of *WRS-85D* allows animals missing zygotic *WRS-85D* function to survive to late larval–pupal stages.
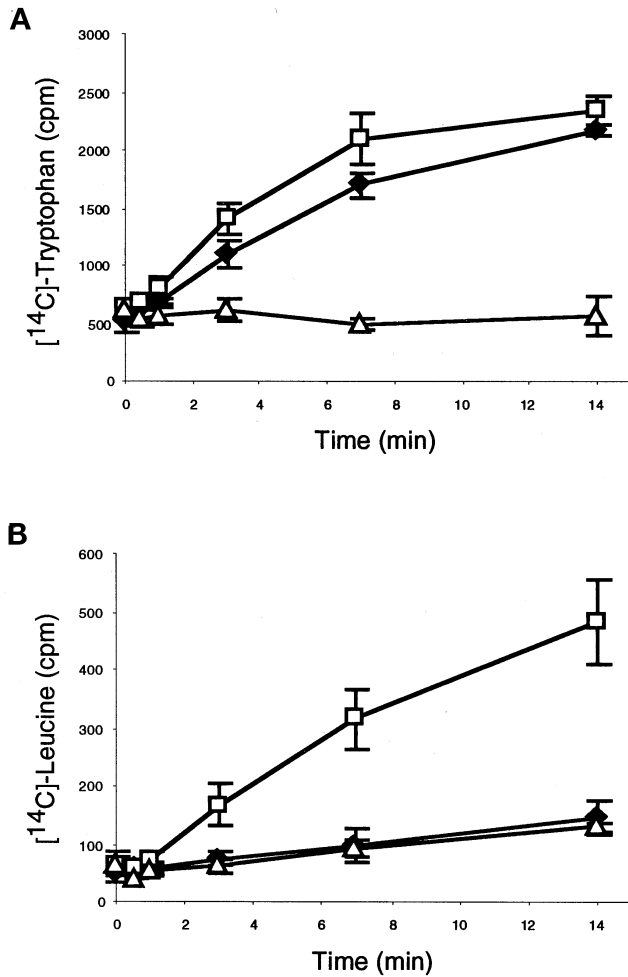
A

B

Figure 4. WRS-85D has aminoacylation activity. Bacterially purified WRS-85D was incubated with total tRNA and [14C]L-tryptophan (A) or [14C]L-leucine (B). Protein purified from cells containing the expression vector pTrcHisB alone was used as a negative control. Reticulocyte lysate was used as a positive control. ♦, WRS-85D; □, reticulocyte lysate; △, pTrcHisB. Data are the mean ± SD of three independent experiments for each sample.

Table 1. WRS-85D expression in excision alleles

Allele	Original name	Viability	WRS-85D protein in salivary gland
WRS-85D[1]	*l(3)3559*	Lethal	−
WRS-85D[2]	*l(3)4410*	Lethal	−
WRS-85D[3]	*3559exc35*	Lethal	−
WRS-85D[4]	*3559exc50*	Lethal	−
WRS-85D[5]	*3559exc74*	Lethal	−
WRS-85D[6]	*4410exc14*	Lethal	−
WRS-85D[7]	*4410exc18*	Lethal	−
WRS-85D[8]	*4410exc28B*	Lethal	−
WRS-85D[9]	*4410exc41*	Lethal	−
Wild-type	*3559exc25*	Viable	+
Wild-type	*4410exc30*	Viable	+

Drosophila Genome Project Expressed Sequence Tags database for cDNAs with homology to aminoacyl-tRNA synthetase genes. We found multiple expressed sequence tags (ESTs) corresponding to each aminoacyl-tRNA synthetase. We then used these ESTs to search GenBank and selected the EST with the highest homology to each aminoacyl-tRNA synthetase. Once we obtained cDNAs corresponding to each of the 20 aminoacyl-tRNA synthetases (Genome Systems, St. Louis, MO; Research Genetics, Huntsville, AL), we sequenced the 5′ ends of each clone and verified that we had indeed obtained the correct cDNA.

To determine the expression patterns of each *Drosophila* aminoacyl-tRNA synthetase, we prepared antisense RNA probes for each gene and performed in situ hybridizations to whole-mount embryos. The experiment was carried out in parallel for all 20 genes to eliminate any potential experimental variation in transcript detection. The results are shown in Figure 5 and Table 2. Transcripts were detected in embryos with all 20 probes. Besides *WRS-85D*, two other aminoacyl tRNA synthetases, seryl-tRNA synthetase and alanyl-tRNA synthetase had elevated levels of expression in the salivary gland primordia that persisted throughout embryogenesis (Figure 5, D–I). Threonyl-tRNA synthetase showed a slight elevation in expression in the salivary gland primordia (Figure 5K), which increased at later stages (Figure 5L). Several other aminoacyl-tRNA synthetase genes showed transiently increased levels of expression in the salivary gland primordia that persisted from approximately embryonic stage 11–12 (stages according to Campos-Ortega and Hartenstein, 1997). Some aminoacyl-tRNA synthetase genes showed no increase in expression in the salivary gland or salivary gland primordia (Figure 5, M–O), and at least two others had high-level expression in other cells, specifically in the embryonic muscle precursors (Figure 5, P–R).

Other Aminoacyl-tRNA Synthetases in Drosophila Have Elevated Expression Levels in the Salivary Gland

A possible explanation for the elevated levels of *WRS-85D* expression in the salivary gland secretory cells is that these cells may synthesize very high levels of protein compared with most cells in the embryo. To accommodate increased levels of protein production in the salivary gland, genes encoding enzymes such as aminoacyl-tRNA synthetases that are required for protein synthesis might be expressed to elevated levels. We thus decided to examine the expression pattern of all 20 aminoacyl-tRNA synthetases. To obtain probes for these genes, we first searched the Berkeley
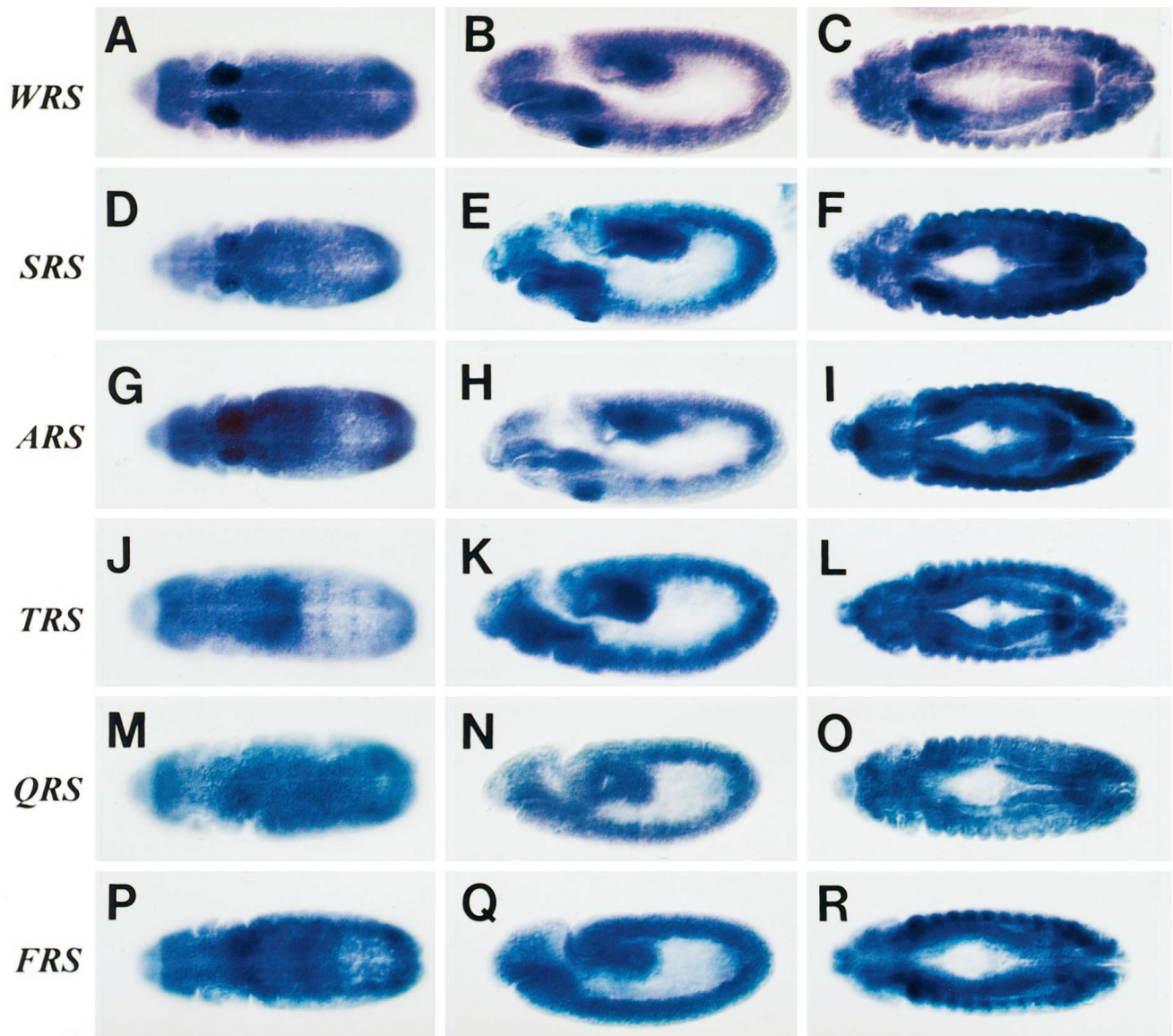
Figure 5. A limited number of aminoacyl-tRNA synthetases show elevated transcript levels in the early salivary gland. Embryos have been hybridized with digoxygenin-labeled antisense RNA probes to different *Drosophila* aminoacyl-tRNA synthetase genes. (A–C) *WRS-85D*; (D–F) *seryl-tRNA synthetase*; (G–I) *alanyl-tRNA synthetase*; (J–L) *threonyl-tRNA synthetase*; (M-O) *glutaminyl-tRNA synthetase*; (P–R) *phenylalanyl-tRNA synthetase*. Embryos shown in the left column are stage 11 ventral views. Embryos in the middle column are stage 11 lateral views. Embryos in the right column are stage 13 ventral views.

DISCUSSION

We have identified a tryptophanyl-tRNA synthetase gene (*WRS-85D*) whose expression is dependent on activity of the homeotic gene *Scr* using an enhancer trap screen for genes regulated by *Scr. Scr* is required for salivary gland formation in *D. melanogaster*. The identity of *WRS-85D* was confirmed by strong homology with mammalian TrpRS, by conservation of HVGH and KMSAS motifs, and by the ability of recombinant WRS-85D protein to aminoacylate tRNA^{Trp}. We have shown that *WRS-85D* is essential for embryos to survive to adulthood. In addition, we found that a defining feature of *WRS-85D* is its elevated levels in the embryonic salivary gland. Finally, we isolated the remaining 19 aminoacyl-tRNA synthetases and found, by in situ hybridization, accumulation of three other aminoacyl-tRNA synthetases, seryl-, alanyl-, and threonyl-tRNA synthetases, in the salivary gland. However, none approached the level of *WRS-85D*.

Table 2. Expression of aminoacyl-tRNA synthetases during embryogenesis

Expression pattern during embryogenesis	Aminoacyl-tRNA synthetases
Highly and persistently elevated in the salivary gland	WRS, ARS, SRS
Moderately and persistently elevated in the salivary gland	TRS
Transiently elevated in the salivary gland	RRS, GRS, IRS, YRS, VRS, NRS, DRS, KRS
Elevated levels in other tissues	HRS, FRS
Equivalent levels in all embryonic tissues	CRS, QRS, ERS, LRS, MRS, PRS

Aminoacyl-tRNA synthetases catalyze the ligation of an amino acid to its cognate tRNA. This reaction requires ATP and occurs in a two-step process with the formation of an enzyme-bound aminoacyladenylate (E:aa~AMP) intermediate, followed by esterification of the activated amino acid to the tRNA and release of AMP (Meinnel *et al.*, 1995). Aminoacyl-tRNA synthetases are divided into two classes. Class I enzymes contain HVGH and KMSAS signature motifs, which help stabilize the transition state during formation of the aminoacyladenylate (Arnez and Moras, 1997). These housekeeping enzymes are presumably expressed in all cells, which raises the question of why four synthetases are expressed at elevated levels in the developing salivary gland. A simple explanation is that the salivary gland, a secretory organ, requires elevated levels of aminoacyl-tRNA synthetases to accommodate high levels of protein synthesis. A prominent example is the set of glue proteins, secreted by the third instar larval salivary gland, which allow the pupae to adhere to solid substrates in preparation for metamorphosis. Because based on β-gal expression from the P-element insertions, WRS-85D expression is maintained at high levels in the salivary gland throughout larval life (our unpublished results), WRS-85D may be important for the high level of synthesis of the glue proteins. We analyzed the amino acid use among the seven *D. melanogaster* glue proteins for which sequence was available (NG-1, NG-2, SGS-3, SGS-4, SGS-5, SGS-7, and SGS-8; GenBank accession numbers 134473, 730133, 134467, 1711388, 134470, 72268, and 134472, respectively) and found that the amino acids alanine, serine, and threonine are relatively abundant in these proteins, with mean frequencies of 6.4, 8.1, and 16.2%, respectively. Thus, the elevated expression levels of alanyl-, seryl-, and threonyl-tRNA synthetases observed in the salivary gland may correspond to the production of proteins enriched in the cognate amino acids. However, tryptophan had the lowest amino acid use frequency among all 20 amino acids, with a mean frequency of 0.7%. Similarly, among proteins known to be expressed in the embryonic salivary gland, including αPS3 integrin, CREB, CRUMBS, DHR78, fork head, huckebein, semaphorin II, and sulfurylase (GenBank accession numbers 2914733, 345483, 103119, 1036839, 120228, 743794, 436557, and 2073406, respectively), the mean trp composition is 0.7%. Although this analysis does not rule out the possibility of an undiscovered trp-rich salivary gland protein in *Drosophila*, it suggests that there may be a different requirement for the elevated levels of tryptophanyl-tRNA synthetase in the salivary gland.

Tryptophanyl-tRNA synthetases possess unusual properties in other eukaryotic systems. One of the most striking is the up-regulation of TrpRS by interferon gamma (IFN-γ) in a number of cell types, including several human cell culture lines (Kisselev *et al.*, 1993; Reano *et al.*, 1993). IFN-γ is a cytokine that mediates both antiproliferative and antiviral effects (Burke *et al.*, 1995). Human *WRS* contains IFN-stimulating response elements and IFN-γ activation sites, which are *cis*-acting elements upstream of the start of transcription that are bound by transcription factors activated by IFN stimulation (Frolova *et al.*, 1993; Strehlow *et al.*, 1993; Eilers *et al.*, 1994; Tolstrup *et al.*, 1995). In *WRS-85D*, we found the sequences TTTCT-GTGAA, a very close match to the IFN-γ activation site consensus TTNCNNNA, and CCAATCG in inverse orientation, a perfect match to the Y-box (CT-GATTGG), which is necessary for IFN-γ induction of major histocompatibility complex class II genes (Tolstrup *et al.*, 1995). Mammalian TrpRS transcripts are also known to be alternatively spliced and polyadenylated, leading to differences in transcript size of 800 bp (Pajot *et al.*, 1994; Tolstrup *et al.*, 1995; Shen *et al.*, 1996; Turpaev *et al.*, 1996). Likewise, we also isolated an alternatively spliced form of *WRS-85D* in which the fourth intron is retained; this mRNA is 1 kb shorter and contains an alternative polyadenylation signal (Figure 2A).

Drosophila secrete both antibacterial and antifungal peptides as part of their immune response to infection. A sequence with similarity to the mammalian IFN response element has been shown to positively regulate the promoter of the *Drosophila* gene for the antibacterial peptide diptericin (Georgel *et al.*, 1995). Additionally, when larvae containing a transgene of the antifungal peptide drosomycin promoter fused to green fluorescnet protein were exposed to a concentrated fungal solution, green fluorescent protein expression was induced in the salivary gland and other tissues (Ferrandon *et al.*, 1998). However, the question of whether a link exists between the induction of the immune response in *Drosophila* and the expression of the *Drosophila* TrpRS *WRS-85D* remains to be answered.

The functional relationship between up-regulation of a housekeeping gene and the pleiotropic effects of IFN-γ is unknown. A possible link is that IFN-γ also up-regulates expression of the enzyme indoleamine 2,3-dioxygenase (IDO), which catabolizes tryptophan (Pfefferkorn et al., 1986). The growth-inhibitory effects of IFN-γ on intracellular parasites have been attributed to the induction of IDO and subsequent depletion of tryptophan (Byrne et al., 1986; Pfefferkorn et al., 1986). In cell lines, the antiproliferative effect of IFN-γ was shown to be most potent in lines in which IDO was induced; the addition of tryptophan to the medium reversed these antiproliferative effects (Burke et al., 1995). It has been proposed that the up-regulation of WRS by IFN-γ may allow host cells to continue protein synthesis in an environment with depleted levels of tryptophan (Flohr et al., 1992). Alternatively, WRS may help sequester TRP into a form that cannot be used for parasite proliferation. The effects of IDO have recently been implicated in prevention of fetal rejection in mice by showing that an IDO inhibitor increases fetal rejection (Munn et al., 1998). It will be interesting to determine whether WRS levels are altered at the maternal–fetal interface and whether addition of exogenous tryptophan also causes an increase in fetal rejection.

All aminoacyl-tRNA synthetases can catalyze the formation of dinucleotide oligophosphates by the back-reaction of ATP or ADP with the E:aa~AMP intermediate to produce AppppA (Ap$_4$A) or AppppA (Ap$_3$A) (Goerlich et al., 1982). These molecules are called "alarmones," in reference to the molecules in prokaryotes that accumulate in response to metabolic stress, such as amino acid starvation. In prokaryotes, alarmones initiate cellular changes such as the stringent response, which results in the shutdown of rRNA and tRNA synthesis (Gallant, 1979). Among its effects in eukaryotic systems, Ap$_n$A is associated with nuclear functions such as stimulation of DNA synthesis, mitogenic activity, and activation of transcription (Kisselev et al., 1998). Treatment of cell lines with IFN-γ increases the levels of intracellular Ap$_3$A through induction of WRS expression (Merkulova et al., 1994; Vartanian et al., 1996). However, unlike the majority of aminoacyl-tRNA synthetases, mammalian WRS only synthesizes Ap$_3$A.

Furthermore, mutations in a putative tumor suppressor gene, FHIT, which is an Ap$_3$A hydrolase, are found in esophageal, stomach, and colon carcinomas (Barnes et al., 1996; Ohta et al., 1996). Although the cellular pathways of FHIT are not understood, FHIT-substrate–bound complex is likely to be the signaling form of the enzyme (Pace et al., 1998). Whether there is a functional relationship between the antiproliferative effects of IFN-γ and the induction of WRS, an enzyme that can synthesize Ap$_3$A, and the function of FHIT, a protein that potentially requires Ap$_3$A for its activity

as a tumor suppressor, remains to be seen. The recent identification of the Drosophila FHIT homologue will allow us to study the interaction between WRS-85D and FHIT (Pekarsky et al., 1998).

The elevated levels of WRS-85D in the salivary gland do not indicate a requirement for synthesis of proteins enriched in tryptophan. However, WRS-85D may be involved in noncanonical functions, such as immune response and control of cell growth. Because Drosophila is highly amenable to genetic analysis, and gene function can be studied within the context of an organ, the salivary gland is a useful model system to determine the roles of tryptophanyl-tRNA synthetase.

ACKNOWLEDGMENTS

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